A soluble form of CD4 (T4) protein inhibits AIDS virus infection

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CD4 (T4) is a glycoprotein of relative molecular mass 55,000 (M_r 55K) on the surface of T lymphocytes which is thought to interact with class II MHC (major histocompatibility complex) molecules, mediating efficient association of helper T cells with antigen-bearing targets[1-3]. The CD4 protein is also the receptor for HIV, a T-lymphotropic RNA virus responsible for the human acquired immune deficiency syndrome (AIDS) (refs 4–7). To define the mechanisms of interaction of CD4 with the surface of antigen-presenting cells and with HIV, we have isolated the CD4 gene and expressed this gene in several different cellular environments[7,8]. Here we describe an efficient expression system in which a recombinant, soluble form of CD4 (sCD4) is secreted into tissue culture supernatants. This sCD4 retains the structural and biological properties of CD4 on the cell surface, binds to the envelope glycoprotein (gp110) of HIV and inhibits the binding of virus to CD4⁺ lymphocytes, resulting in a striking inhibition of virus infectivity.

The CD4 molecule is comprised of an N-terminal hydrophobic signal sequence, four extracellular domains with homology to immunoglobulin variable and joining regions, a hydrophobic transmembrane domain, and a highly charged cytoplasmic segment[8,9]. To obtain large quantities of the extracellular segment of CD4, we introduced a termination codon into the CD4 complementary DNA[8] at the extracellular boundary of the transmembrane domain, expecting that the truncated protein would be secreted in a soluble form. This sCD4 cDNA was inserted between the SV40 early promoter and the bovine growth hormone (BGH) polyadenylation site (Fig. 1*a*). The sCD4 expression cassette was linked to a mouse dihydrofolate reductase (*dhfr*) expression cassette[10] to permit co-amplification of the sCD4 gene on exposure to increasing concentrations of the *dhfr* inhibitor methotrexate (MTX). This vector, psT4DHFR (Fig. 1*a*), was transfected into the *dhfr*-deficient CHO cell line, DXBll (ref. 11), and transformants were selected and exposed to progressively increasing concentrations of methotrexate[12]. Supernatants from clones were monitored for the expression of sCD4 by one of three independent assays: (1) immunoprecipitation from ^{35}S-labelled cultures with CD4-specific monoclonal antibodies; (2) Western blot analysis using a rabbit anti-CD4 polyclonal antibody developed against a denatured CD4 protein produced in bacteria; and (3) competition enzyme-linked immunosorbent assay (ELISA) performed with immobilized sCD4.

^{35}S-methionine- and ^{35}S-cysteine-labelled sCD4 from culture supernatants was specifically immunoprecipitated by each of six anti-CD4 monoclonal antibodies but not by control antibodies (Fig. 1*b*). In contrast, when culture supernatants were analysed under denaturing conditions by Western blot analysis, sCD4 was not recognized by any of the six anti-CD4 monoclonal

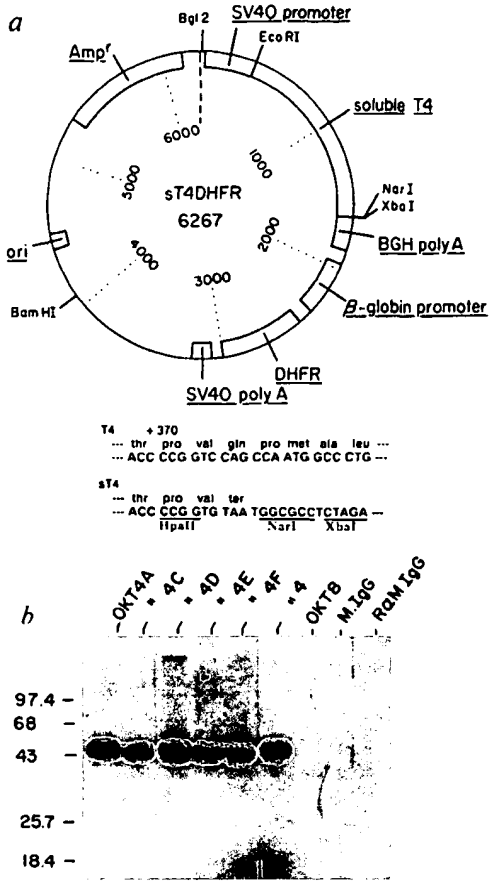

Fig. 1 *a*, Plasmid psT4DHFR is a pUC18 derivative containing base pairs (bp) 1–1,257 of the CD4 cDNA clone pT4B[8] which encodes the leader and extracellular segment of CD4. This sCD4 cDNA is inserted between an SV40 early promoter and a synthetic linker containing a TAA termination codon (inset) followed by the polyadenylation region of the bovine growth hormone gene. The sCD4 expression cassette is linked to a mouse *dhfr* expression cassette consisting of the β-globin promoter, mouse *dhfr* coding sequence and the SV40 polyadenylation region. *b*, Epitope characterization of sCD4. Cell lines were metabolically labelled with ^{35}S-methionine and cysteine and the supernatants were immunoprecipitated with monoclonal antibodies to CD4 (OKT4, T4A, T4C-F), CD8, and control antibodies (mouse IgG (M. IgG) and rabbit anti-mouse IgG (RαMIgG)). Regular molecular masses (M_r) are given in thousands.

Methods. *a*, Plasmid psT4DHFR was constructed in pUC18 and contains the following sequence elements isolated from previously reported plasmids: SV40 early promoter[10], SV40 poly(A) early region[10], bovine growth hormone poly(A) region[10], mouse β-globin promoter[14], mouse *dhfr* coding region[15] and bp 1–1257 of the CD4 cDNA clone pT4B[8]. The full-length CD4 cDNA was truncated at a *Hpa*II site (bp 1,252) and ligated to a synthetic linker (inset) which restored bp 1,253–1,257 of the CD4 coding sequence and placed a TAA termination codon after bp 1,257. DXB-11 cells, a *dhfr*⁻ CHO cell line[11], were transfected by calcium phosphate precipitation[16] with 10 or 30 μg of psT4DHFR one day after seeding. Transformants were selected in nucleoside-free medium and the transforming sequences were amplified through rounds of increasing MTX concentrations[12]. *b*, Cultures containing 1×10^6 cells per 60 mm culture dish were labelled for 16 h at 37 °C in 1.5 ml methionine and cysteine free F12 medium containing ITS (Collaborative), glutamine, 170 μCi ml^{-1} ^{35}S-methionine and 30 μCi ml^{-1} ^{35}S-cysteine (ICN). Clarified media was precleared, incubated with 5 μg OKT4, T4A, T4C-T4F, OKT8 (P. Rao, Ortho), mouse IgG (Cooper), or rabbit anti-mouse IgG (Cooper) for 30 min at 4 °C, then incubated with protein A-Sepharose beads. The beads were washed twice, boiled for 5 min in 20 μl sample buffer and analysed by electrophoresis through a 12.5% SDS-polyacrylamide gel under reducing conditions.

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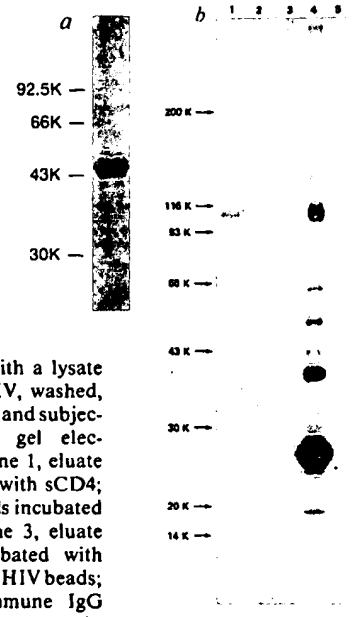

Fig. 2 *a*, SDS-PAGE profile of purified sCD4. Relative molecular masses (M_r) are given in kilodaltons. *b*, Coprecipitation of HIV gp110 with sCD4. Supernatant containing sCD4 and control supernatant from untransformed DXB-11 cells were adsorbed to Sepharose beads coated with OKT4, control antibody MOPC141 (isotype matched to OKT4), or human anti-HIV IgG. The beads were washed, mixed with a lysate of ^{35}S-methionine-labelled HIV, washed, and bound material was eluted and subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Lane 1, eluate from OKT4 beads incubated with sCD4; lane 2, eluate from OKT4 beads incubated with control supernatant; lane 3, eluate from MOPC141 beads incubated with sCD4; lane 4, eluate from anti-HIV beads; lane 5, eluate from non-immune IgG beads. Relative molecular masses are given.

Methods. *a*, Purified sCD4 (3 μg) was electrophoresed through a 12.5% polyacrylamide gel and visualized by silver stain. *b*, A lysate of extracellular HIV was internally labelled with ^{35}S-methionine and ^{35}S-cysteine as previously described[6]. Sepharose beads coupled to OKT4 monoclonal antibody, MOPC141 paraprotein (isotype matched to OKT4), human anti-HIV IgG and non-immune human IgG were prepared as previously described[6]. OKT4 beads and MOPC141 beads (10 μl) were incubated for 2 h at 4 °C with ~5 μg of sCD4, or control supernatant from untransformed DXB-11 cells, then washed once with lysing buffer. The ^{35}S-labelled HIV (preadsorbed) was added and the beads were incubated for 3 h at 4 °C. Washing, elution of bound material and SDS-PAGE under reducing conditions were performed as previously described[6].

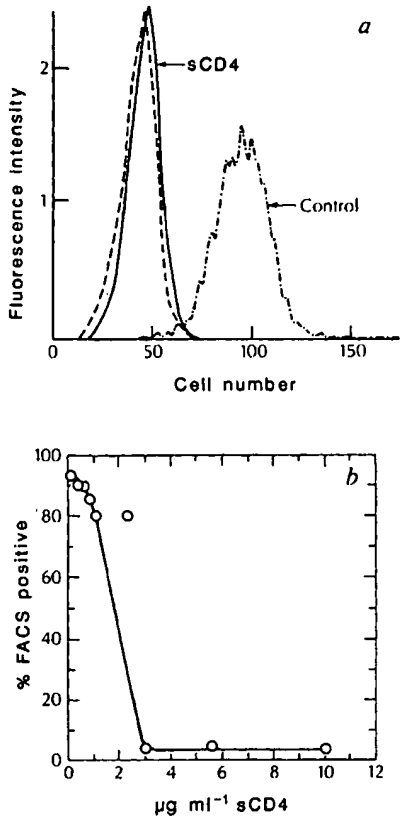

Fig. 3 *a*, sCD4 inhibits HIV binding to CD4$^+$ CEM T cells. Cells were incubated with buffer (- - - -), HIV precincubated with sCD4 (——), or with HIV preincubated with concentrate control supernatant from untransformed DXB-11 (—·—·), washed, exposed to fluorescein-conjugated anti-HIV antibody, and analysed by cytofluorometry as previously described[11]. A fluorescence histogram (cell number against fluorescence intensity) is shown. *b*, Plot of percentage of positive cells by flow cytometry versus concentration of sCD4.

antibodies (which recognize only native protein), but was readily detected by rabbit anti-CD4 polyclonal serum raised against a denatured form of CD4 produced in bacteria (not shown). Several clones synthesize >3 pg of sCD4 per cell in 24 hours, giving ~40 mg of sCD4 per litre in high-density suspension cultures over 4 days. We have purified large quantities of sCD4 to >95% purity (Fig. 2*a*), and this purified protein shows the same pattern of antibody reactivity as described for sCD4 in culture supernates. These data suggest that sCD4 maintains a configuration analogous to the native extracellular domain of CD4 on the lymphocyte surface.

We next examined whether sCD4 associates with HIV envelope glycoprotein gp110. Purified sCD4 (~5 μg) was adsorbed to Sepharose beads coated with OKT4 or control antibody. OKT4 associates with the CD4 molecule without inhibiting the ability of CD4 to interact with virus[4-7]. The beads were mixed with a lysate of ^{35}S-methionine-labelled HIV. Only the 110K envelope glycoprotein is coprecipitated by the complex of sCD4 with OKT4 (Fig. 2*b*, lane 1); no viral proteins are precipitated by OKT4 beads without sCD4 or in the presence of control supernatants from the untransfected CHO cells (Fig. 2*b*, lane 2). Furthermore, no viral protein is precipitated if Sepharose beads coated with control mouse immunoglobulin (isotype matched to OKT4) are incubated with sCD4 (Fig. 2*b*, lane 3). Thus the soluble CD4 protein, without other T-cell surface components can specifically associate with the envelope glycoprotein of the AIDS virus.

To examine whether sCD4 abolishes HIV binding to the surface of CD4$^+$ cells, CEM T cells (which carry CD4) were exposed to HIV in the presence or absence of sCD4. After viral adsorption, the cells were washed, exposed to fluorescein-conjugated anti-HIV antibody, and analysed by flow cytometry (Fig. 3*a*)[6,7]. HIV binds efficiently to CEM cells, but if the virus is preincubated with sCD4 the binding is abolished; 3 ng of purified sCD4 is sufficient to inhibit the binding of 100 ng of viral protein (Fig. 3*b*). We estimate that if the envelope glycoprotein comprises 5% of the total viral protein, then a 2:1 molar ratio of CD4 to gp120 can completely inhibit HIV binding to CD4$^+$ cells.

Finally, we have examined the ability of soluble CD4 to inhibit the infection of CD4$^+$ cells by HIV. Phytohaemagglutinin (PHA)-stimulated human lymphocytes were exposed to serial ten-fold dilutions of an HIV inoculum in the presence or absence of sCD4, washed and plated in microculture. The frequency of infected culture was determined using an immunoassay 4, 8 and 12 days after exposure to virus[13] (Fig. 4*a*). We define the infectious virus titre, ID-50, as the reciprocal of the dilution required to infect 50% of the exposed cell cultures at day 12 (Fig. 4*b*). In the absence of sCD4, the ID-50 observed with our viral inoculum is ~10^5. However, in the presence of 8 μg ml^{-1} purified sCD4, the infectivity is diminished to an ID-50 of $10^{1.5}$ (Fig. 4*b*). This dramatic reduction in infectivity is observed throughout the course of infection. As a control for nonspecific inhibition or toxic effects, sCD4 was added to cultures 18 h after the initial exposure to virus. These cultures show only a 1 log inhibition in the ID-50 (Fig. 4*b*), probably resulting from inhibition of virus spread after the initial inoculation. Thus, the inhibition of virus infection by sCD4 is likely to be a result of

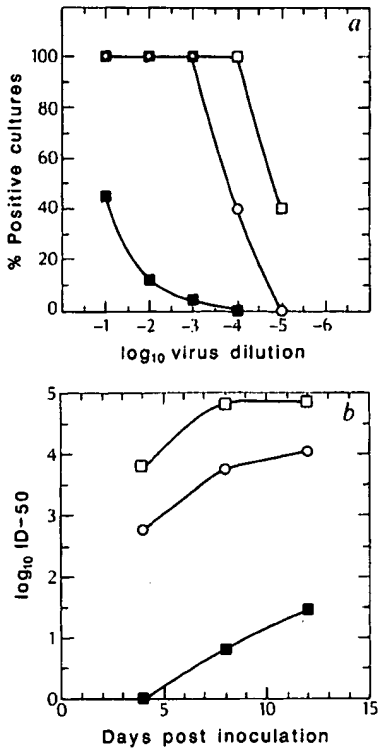

Fig. 4 Inhibition of HIV infectivity by sCD4. *a*, Plot of percentage of cultures positive for HIV at day 8 versus dilution of virus inoclum; *b*, Plot of ID-50 (reciprocal of virus dilution at which 50% of cultures are positive) at days 4, 8 and 12. Infectivity titration of an HIV inoculum (ID-50 assay) was performed as previously described[17]. Briefly, serial 10-fold dilutions of virus inoculum are incubated with indicator cells (PHA-stimulated human lymphocytes) for 18 h. The cells are then washed and plated in microculture (1×10^5 cells per culture, 10 cultures per dilution). At days 4, 8 and 12, supernatants are tested for HIV by the antigen capture assay[13]. ID-50 titrations were performed in media containing 8.6 μg ml^{-1} sCD4 which was added to the HIV dilution 30 min before inoculation of cells and maintained in the culture media throughout the experiment (■—■), or in media containing sCD4 introduced after the initial 18 h inoculum (○—○) (delayed addition control), or in media without sCD4 (□—□), control).

the specific association of sCD4 with gp110, inhibiting the interaction of virus with CD4$^+$ cells. We estimate that our viral preparations of 10^4 infectious particles per ml actually contain 10^8 particles per ml. If each particle contains 1,000 envelope glycoproteins, the 3.5 log inhibition we observe is obtained at a ratio of 1,000 CD4 molecules per molecule of envelope glycoprotein.

The ability of sCD4 to bind gp110 and inhibit viral infection *in vitro* immediately suggests the potential use of sCD4 as an antiviral agent in AIDS patients. Although significant variance exists among the different HIV isolates, each appears to use CD4 as a cellular receptor. Thus, sCD4 is likely to be a universal inhibitor of viral infection. Although sCD4 is extremely effective *in vitro*, its use in man will depend on the pharmacokinetics of sCD4, its immunogenicity, its effects on the cellular immune response and the clinical significance of inhibiting viral spread in infected individuals. Whatever the efficacy of sCD4 as a therapeutic in AIDS, this reagent should allow a more precise understanding of the structural basis for the association of CD4 with the HIV envelope glycoprotein and with the surface of antigen-presenting cells.

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Soluble CD4 molecules neutralize human immunodeficiency virus type 1

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Human immunodeficiency virus (HIV) infection can bring about total collapse of the immune system[1,2] by infecting helper T lymphocytes which express CD4, the molecule which mediates interaction between the cell surface and viral envelope glycoprotein gp120 (refs 3–10). HIV apparently escapes the effects of neutralizing antibodies *in vivo* by generating new variants which must still interact with CD4 to maintain a cycle of infection[11–14]. One route to block HIV infection, therefore, could use solubilized CD4 protein to inhibit attachment of the virus to its target cell. We have used recombinant DNA techniques to generate soluble forms of CD4, and show here that these are potent inhibitors of HIV infection *in vitro*.

Two different chimaeric genes based on immunoglobulin-expression systems were made (Fig. 1). The construct HT4-Y1 codes for the N-terminal immunoglobulin-like region and three following domains of CD4 which comprise the whole extracellular portion; the other, HT4-X6, codes for the immunoglobulin-like and second domains only[15,16]. In both cases the C-terminal part of the protein consists of the immunoglobulin κ light-chain constant region.

The introduction of these constructs into myeloma cells (Fig. 2) resulted in the secretion of the two chimaeric proteins of predicted apparent molecular weight: HT4-Y1 and HT4-X6 transformants produced molecules of relative molecular mass (M_r) 60,000 (60K) and 30K respectively, as detected by Western blotting with anti-κ antibodies (Fig. 2*a*). Furthermore, immunoprecipitations of the supernatants with two different monoclonal anti-CD4 antibodies (Fig. 2*b*) showed that these secreted proteins retained at least some of their original conformation.

In addition, the soluble CD4 proteins could bind at low concentration to purified HIV-envelope protein gp120 in solid-phase assays, consistent with a high-affinity interaction[10] (Fig. 3). The lower intensity of the signal generated by the 'complete' CD4 (Y124) compared to the truncated one (X6-10) is probably due to a concentration difference in the supernatants (20 and